

BIRCH, STEWART, KOLASCH & BIRCH, LLP

INTELLECTUAL PROPERTY LAW  
8110 GATEHOUSE ROAD  
SUITE 500 EAST  
FALLS CHURCH, VA 22042-1210  
U S A  
(703) 205-8000

FAX. (703) 205-8050  
(703) 698-8590 (G IV)

e-mail mailroom@bskb.com  
web http://www.bskb.com

CALIFORNIA OFFICE  
COSTA MESA, CALIFORNIA

715 U.S.  
00/60/90  
RELL C BIRCH  
RAYMOND C STEWART  
JOSEPH A KOLASCH  
JAMES M. SLATTERY  
BERNARD L. SWEENEY\*  
MICHAEL K. MUTTER  
CHARLES GORENSTEIN  
GERALD M. MURPHY, JR.  
LEONARD R. SVENSSON  
TERRY L. CLARK  
ANDREW D. MEIKLE  
MARC S. WEINER  
JOE MCKINNEY MUNCY  
ROBERT J. KENNEY  
DONALD J. DALEY  
JOHN W. BAILEY  
JOHN A. CASTELLANO, III  
GARY D. YACURA

OF COUNSEL  
HERBERT M. BIRCH (1905-1996)  
ELLIOT A. GOLDBERG\*  
WILLIAM L. GATES\*  
EDWARD H. VALANCE  
RUPERT J. BRADY (RET)\*  
F. PRINCE BUTLER  
FRED S. WHISENHUNT

\*ADMITTED TO A BAR OTHER THAN VA

THOMAS S. AUCHTERLONIE  
JAMES T. ELLER, JR.  
SCOTT L. LOWE  
MARK J. NUEL, Ph.D.  
D. RICHARD ANDERSON  
PAUL C. LEWIS  
MARK W. MILSTEAD\*  
JOHN CAMPA\*  
RICHARD J. GALLAGHER  
REG. PATENT AGENTS  
FREDERICK R. HANDREN  
MARYANNE ARMSTRONG, Ph.D.  
MAKI HATSUMI  
MIKE S. RYU  
CRAIG A. McROBBIE  
GARTH M. DAHLEN, Ph.D.  
LAURA C. LUTZ  
ROBERT E. GOOZNER, Ph.D.  
HYUNG N. SOHN  
MATTHEW J. LATTIG  
ALAN PEDERSEN-GILES  
JUSTIN D. KARJALA  
C. KEITH MONTGOMERY  
TIMOTHY R. WYCKOFF  
HERMES M. SOYEZ, Ph.D.  
KRISTIL RUPERT, Ph.D.

06/09/00  
09/590375  
1c829 U.S. PTO



Date: June 9, 2000  
Docket No.: 2173-0120P

Assistant Commissioner for Patents  
Box PATENT APPLICATION  
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): ENDO, Keiji  
IGARASHI, Kazuaki; HAYASHI, Yasuhiro  
HAGIHARA, Hiroshi; OZAKI, Katsuya

For: MUTANT ALPHA-AMYLASES

Enclosed are:

- ☒ A specification consisting of 55 pages
- ☒ 3 sheet(s) of Formal drawings
- ☒ An assignment of the invention
- ☒ Certified copy of Priority Document(s)
- ☒ Executed Declaration ☒ Original ☐ Photocopy
- ☐ A verified statement to establish small entity status under 37 CFR 1.9 and 37 CFR 1.27
- ☒ Preliminary Amendment
- ☐ Information Disclosure Statement, PTO-1449 and reference(s)

X Other 3- Budapest Treaty Deposit REceipts w/English translation

The filing fee has been calculated as shown below:

LARGE ENTITY				SMALL ENTITY	
FOR	NO. FILED	NO. EXTRA	RATE FEE		RATE FEE
BASIC FEE	***** ***** *****	***** ***** *****	***** ***** \$690.00 *****	or	**** **** \$345.00 ****
TOTAL CLAIMS	13 - 20 =	0	x18 =\$ 0.00	or	x 9 = \$ 0.00
INDEPENDENT	2 - 3 =	0	x78 =\$ 0.00	or	x 39 = \$ 0.00
MULTIPLE DEPENDENT CLAIM PRESENTED <u>yes</u>			+260 = \$260.00	or	+130 = \$ 0.00
			TOTAL \$ 950.00		TOTAL \$ 0.00

X A check in the amount of \$ 990.00 to cover the filing fee and recording fee (if applicable) is enclosed.

— Please charge Deposit Account No. 02-2448 in the amount of \$\_\_\_\_\_. A triplicate copy of this transmittal form is enclosed.

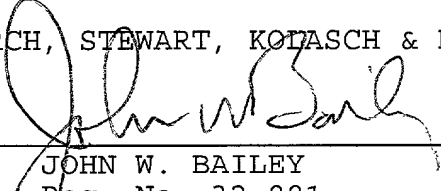
— No fee is enclosed.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. 1.16 or under 37 C.F.R. 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOTASCH & BIRCH, LLP

By

  
JOHN W. BAILEY  
Reg. No. 32,881  
P. O. Box 747

Falls Church, Virginia 22040-0747

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: ENDO, Keiji et al  
Appl. No.: NEW Group: UNKNOWN  
Filed: June 9, 2000 Examiner: UNKNOWN  
For: MUTANT ALPHA-AMYLASES

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, DC 20231

June 9, 2000

Sir:

The following preliminary amendments and remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE CLAIMS:

Please amend the claims as follows:

**CLAIM 5:** (Amended) A mutant  $\alpha$ -amylase obtained by introducing a mutation into an  $\alpha$ -amylase having the amino acid sequence set forth in SEQ ID NO:1 or an  $\alpha$ -amylase having a homology of at least 70% to said amino acid sequence with at least two kinds of

replacement or deletion selected from the replacement or deletion of the amino acid residues respectively corresponding to the 11<sup>th</sup> Tyr, 16<sup>th</sup> Glu, 49<sup>th</sup> Asn, 84<sup>th</sup> Glu, 144<sup>th</sup> Ser, 167<sup>th</sup> Gln, 169<sup>th</sup> Tyr, 178<sup>th</sup> Ala, 188<sup>th</sup> Glu, 190<sup>th</sup> Asn, 205<sup>th</sup> His and 209<sup>th</sup> Gln [set forth in Claim 1], and the replacement of the amino acid sequence set forth in any one of Claims 2 to 3 [4] combined with each other.

**CLAIM 7:** Line 2, change "any one of Claims 1 to 6" to --Claim 1--

**CLAIM 10:** Line 2, change "any one of Claims 1 to 6" to --Claim 1--

REMARKS

The amendment to the claims is to delete the improper multiple dependencies in order to place the application in better form prior to examination.

Entry of the present amendment and favorable action on the above-identified application is respectfully requested.


If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees

Docket No. 2173-0120P

required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17;  
particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By   
John W. Bailey, #32,881

JWB/dpt  
2173-0120P

P.O. Box 747  
Falls Church, VA 22040-0747  
(703) 205-8000

(Rev. 04/19/2000)

TITLE OF THE INVENTION:

MUTANT  $\alpha$ -AMYLASES

BACKGROUND OF THE INVENTION

5 Field of the Invention:

The present invention relates to mutant liquefying alkaline  $\alpha$ -amylases which have excellent heat resistance, and are particularly useful as enzymes for detergents, and genes thereof.

10 Description of the Background Art:

When an  $\alpha$ -amylase [EC.3.2.1.1] is used as an enzyme for detergents, it has heretofore been said that a liquefying alkaline  $\alpha$ -amylase, which can decompose starch at random and is stable to alkali and also to both  
15 chelating component and oxidation bleaching component, is preferred. However, in liquefying amylases, a calcium ion is generally important for maintaining the structure of the enzymes, and the stability thereof is lowered in the presence of a chelating agent. Besides, most of such  
20 enzymes have had the optimum pH in a neutral to weakly acidic range.

Under the foregoing circumstances, the present inventors found that enzymes produced by alkaliphilic *Bacillus* sp. KSM-K38 (FERM BP-6946) and *Bacillus* sp. KSM-  
25 K36 (FERM BP-6945) strains isolated from soil do not show the lowering of activity at all in the presence of a chelating agent at a high concentration by which

deactivation is recognized in the conventional liquefying  
 $\alpha$ -amylases, and have resistance to surfactants and  
oxidizing agents and that they have higher activity on the  
alkaline side compared with the conventional liquefying  $\alpha$ -  
5 amylases and are useful as enzymes for detergents  
(Japanese Patent Application No. 362487/1998.

However, said enzymes exhibit inactivation at a  
temperature of 50°C or higher, and so the heat resistance  
thereof have been somewhat insufficient in view of the  
10 fact that cleaning of clothing and tableware is generally  
conducted at about 10 to 60°C.

#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide  
an  $\alpha$ -amylase which is a liquefying alkaline  $\alpha$ -amylase that  
15 has high activity on the alkaline side and is stable to  
both chelating component and oxidation bleaching component,  
and has excellent heat resistance.

The present inventors have acquired various mutant  
enzymes as to liquefying alkaline  $\alpha$ -amylases and  
20 investigated them. As a result, it has been found that  
when a mutation is introduced into a specified amino acid  
residue in the amino acid sequence (SEQ ID NO:1) of  
amylase derived from KSM-K38, the heat resistance of the  
enzyme is improved without losing its properties such as  
25 resistance to chelating agents and resistance to oxidizing  
agents and high specific activity in an alkaline region,  
and that the heat resistance can be further improved by

combining such mutations.

According to the present invention, there is thus provided a mutant  $\alpha$ -amylase obtained by making replacement or deletion of at least one residue of amino acid residues  
5 respectively corresponding to the 11th Tyr, 16th Glu, 49th Asn, 84th Glu, 144th Ser, 167th Gln, 169th Tyr, 178th Ala, 188th Glu, 190th Asn, 205th His and 209th Gln in the amino acid sequence set forth in SEQ ID NO:1 in an  $\alpha$ -amylase having said amino acid sequence, or an  $\alpha$ -amylase having a  
10 homology of at least 70% to said amino acid sequence.

According to the present invention, there is also provided a mutant  $\alpha$ -amylase obtained by making replacement of a sequence corresponding to 11 to 100 amino acid residues from the amino terminal in the amino acid  
15 sequence set forth in SEQ ID NO:1 in an  $\alpha$ -amylase having said amino acid sequence, or an  $\alpha$ -amylase having a homology of at least 70% to said amino acid sequence by an amino acid sequence of another liquefying  $\alpha$ -amylase corresponding to said sequence of the amino acid residues.

20 According to the present invention, there are further provided genes respectively encoding these mutant  $\alpha$ -amylases, vectors having each of the genes, cells transformed by such a vector, and a production process of these mutant  $\alpha$ -amylases, comprising culturing the  
25 transformed cells.

According to the present invention, there is still further provided a detergent composition comprising any



one of these mutant  $\alpha$ -amylases.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages  
5 of the present invention will become apparent from the  
following description and the appended claims, taken in  
conjunction with the accompanying drawings, in which:

Fig. 1 illustrates a method for preparing a  
recombinant plasmid for the production of  $\alpha$ -amylases  
10 derived from KSM-K38 and KSM-AP1378 strains.

Fig. 2 illustrates a method for introducing a  
mutation into an  $\alpha$ -amylase gene derived from the KSM-38  
strain.

Fig. 3 illustrates a method for replacing an N-  
15 terminal sequence of the  $\alpha$ -amylase gene derived from the  
KSM-38 strain by an N-terminal region of an  $\alpha$ -amylase gene  
derived from the KSM-AP1378 strain.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 The mutant  $\alpha$ -amylases according to the present  
invention are obtained by mutating a gene encoding a  
liquefying alkaline  $\alpha$ -amylase having the amino acid  
sequence set forth in SEQ ID NO:1 or an amino acid  
sequence having a homology of at least 70% to said amino  
25 acid sequence. However, an example where heat resistance  
is improved by deletion and/or replacement of an amino  
acid has also been conducted on the conventional

liquefying  $\alpha$ -amylases. For example, an enzyme obtained by deleting residues from the 177th Arg to the 178th Gly in an enzyme derived from *B. amyloliquefaciens* (J. Biol.

Chem., 264, 18933, 1989) and an enzyme obtained by

5 replacing the 133rd His in an enzyme derived from *B.*

*licheniformis* by Tyr (J. Biol. Chem., 265, 15481, 1990)

have been reported. However, the liquefying alkaline  $\alpha$ -amylases used in the present invention have a low degree of amino acid homology with the conventional liquefying

10 alkaline  $\alpha$ -amylases. In these  $\alpha$ -amylases, a site

corresponding to the residues from the 177th Arg to the

178th Gly has been already deleted, and the amino acid

corresponding to the 133rd His has been already Tyr.

Therefore, the examples of the conventional enzymes cannot

15 be always applied. More specifically, the mutations of the

amino acid sequence for improving the heat resistance in

the present invention are entirely different from the

examples up to the date.

Examples of the liquefying alkaline  $\alpha$ -amylases

20 include an enzyme (Japanese Patent Application No.

362487/1998) derived from a *Bacillus* sp. KSM-K38 (FERM BP-

6946) strain separated from soil by the present inventors

and having the amino acid sequence of SEQ ID NO:1 and an

enzyme (SEQ ID NO:4) (Japanese Patent Application No.

25 362487/1998) derived from *Bacillus* sp. KSM-K36 (FERM BP-

6945) and having a homology of about 95% to the amino acid

sequence of SEQ ID NO:1. Incidentally, the homology of the

amino acid sequence is calculated in accordance with the Lipman-Pearson method (Science, 227, 1435, 1985).

In order to obtain the mutant  $\alpha$ -amylase according to the present invention, a gene encoding a liquefying  $\alpha$ -amylase is first cloned from microorganisms which produce  
5 said liquefying  $\alpha$ -amylase. As a method therefor, a general gene recombination method may be used. For example, the method described in Japanese Patent Application Laid-Open No. 336392/1996 may be used. Examples of the gene  
10 include those set forth in SEQ ID NO:3 and SEQ ID NO:5.

A mutation is then introduced into the gene thus obtained. As a method therefor, any method may be adopted so far as it is a method of site-specific mutation commonly performed. The mutation can be performed, for  
15 example, by using a Site-Directed Mutagenesis System Mutan-Super Express Km kit produced by Takara Shuzo Co., Ltd. An optional sequence of the gene may be replaced by a sequence of another gene corresponding to the optional sequence by using the recombinant PCR (polymer chain  
20 reaction) method (PCR protocols, Academic Press, New York, 1990).

The mutation for improving the heat resistance in the present invention is desirably a mutation in which an amino acid residue corresponding to the 11th Tyr in the  
25 amino acid sequence set forth in SEQ ID NO:1 is replaced by Phe, an amino acid residue corresponding to the 16th Glu by Pro, an amino acid residue corresponding to the

49th Asn by Ser, an amino acid residue corresponding to  
the 84th Glu by Gln, an amino acid residue corresponding  
to the 144th Ser by Pro, an amino acid residue  
corresponding to the 167th Gln by Glu, an amino acid  
5 residue corresponding to the 169th Tyr by Lys, an amino  
acid residue corresponding to the 178th Ala by Gln, an  
amino acid residue corresponding to the 188th Glu by Asp,  
an amino acid residue corresponding to the 190th Asn by  
Phe, an amino acid residue corresponding to the 205th His  
10 by Arg, or an amino acid residue corresponding to the  
209th Gln by Val.

The improvement of heat resistance can also be  
achieved by replacing an amino acid sequence corresponding  
to 11 to 100 amino acid residues from the amino terminal  
15 (Asp) in the amino acid sequence of SEQ ID NO:1 according  
to the present invention, preferably a sequence  
corresponding to amino acid residues from the 1st Asp to  
the 19th Gly, by an amino acid sequence of another  
liquefying  $\alpha$ -amylase corresponding to said sequence of the  
20 amino acid residues.

Examples of said another liquefying  $\alpha$ -amylase used  
in the replacement include an enzyme having the amino acid  
sequence set forth in SEQ ID NO:2. A site of its amino  
acid sequence corresponding to said amino acid residues  
25 from the 1st Asp to the 19th Gly is from the 1st His to  
the 21st Gly. The enzyme is an liquefied  $\alpha$ -amylase  
derived from a *Bacillus* sp. KSM-AP1378 (FERM BP-3048)

strain, and the sequence of the gene is disclosed in Japanese Patent Application Laid-Open No. 336392/1996.

In the mutant  $\alpha$ -amylases according to the present invention, a mutation with at least two kinds of replacement or deletion selected from the replacement or deletion of the above-described various kinds of amino acid residues and the replacement of the amino acid sequences combined with each other is also effective, and mutant enzymes more improved in heat resistance can be obtained by such a combination. More specifically, examples of the combination of mutations include a combination of at least two of the replacement or deletion of the various kinds of amino acid residues, a combination of at least two of the replacement of the amino acid sequence, and a combination of at least two of the replacement or deletion of the amino acid residues and the replacement of the amino acid sequence. Preferably, at least two mutations may be suitably combined from among mutations in which an amino acid residue corresponding to the 49th Asn is replaced by Ser, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 190th Asn by Phe, an amino acid residue corresponding to the 205th His by Arg, and an amino acid residue corresponding to the 209th Gln by Val, and a mutation in which an amino acid sequence corresponding to amino acid residues from the 1st Asp to

the 19th Gly is replaced by an amino acid sequence from the 1st His to the 21st Gly in the amino acid sequence set forth in SEQ ID NO:2.

Examples of the most preferred combination include a  
5 combination of mutations in which an amino acid residue corresponding to the 49th Asn is replaced by Ser, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 190th Asn by  
10 Phe, an amino acid residue corresponding to the 205th His by Arg, and an amino acid residue corresponding to the 209th Gln by Val, and a combination of a mutation in which an amino acid sequence corresponding to amino acid residues from the 1st Asp to the 19th Gly is replaced by  
15 an amino acid sequence from the 1st His to the 21st Gly in the amino acid sequence set forth in SEQ ID NO:2 with a mutation in which an amino acid residue corresponding to the an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 190th  
20 Asn by Phe, or an amino acid residue corresponding to the 209th Gln by Val.

In addition, mutations for improving other properties than the heat resistance, for example, a mutation for more enhancing resistance to oxidizing agents,  
25 in which an amino acid residue corresponding to the 107th Met is replaced by Leu, a mutation for enhancing the detergency of a laundry detergent, in which an amino acid

residue corresponding to the 188th Glu is replaced by Ile, and/or the like may be combined with the above-described mutations.

5 The thus-obtained mutant  $\alpha$ -amylases according to the present invention are improved in stability to heat without losing excellent properties of high resistance to chelating agents, and high specific activity in an alkaline region, and are hence useful for detergents for automatic dish washer, laundry detergents and desizing  
10 agents for fibers.

Such detergents may comprise one or more enzymes selected from debranching enzymes (for example, pullulanase, isoamylase, neopullulanase, etc.),  $\alpha$ -glycosidases, glucoamylases, proteases, cellulases,  
15 lipases, pectinases, protopectinases, pectic acid lyases, peroxidases, laccases and catalases in addition to the above-described mutant  $\alpha$ -amylases.

Further, surfactants such as anionic surfactants, amphoteric surfactants, nonionic surfactants and cationic  
20 surfactants, chelating agents, alkalizing agents, inorganic salts, resoiling preventives, chlorine scavengers, reducing agents, bleaching agents, fluorescent dye solubilizers, perfume bases, caking preventives, enzyme activators, antioxidants, preservatives, coloring  
25 matter, bluing agents, bleaching activators, enzyme stabilizers, phase adjusters, etc., which are commonly incorporated into the classical detergents, may be

incorporated.

The detergent composition according to the present invention can be produced by combining the above-described mutant  $\alpha$ -amylases with the publicly known detergent components described above in accordance with a method known *per se* in the art. The form of the detergent composition may be suitably selected as necessary for the end application intended, and the detergent composition may be provided in the form of, for example, liquid, powder or granules. The detergent composition according to the present invention can be used as a laundry detergent, bleaching detergent, detergent for automatic dish washer, drain cleaner, artificial tooth cleaner or the like. In particular, it can preferably used as a laundry detergent, bleaching detergent or detergent for automatic dish washer.

The mutant  $\alpha$ -amylases according to the present invention may be used as compositions for liquefaction and saccharification of starch and be also caused to act on starch together with one or more enzymes selected from glucoamylase, maltase, pullulanase, isoamylase, neopullulanase, etc.

The mutant  $\alpha$ -amylases according to the present invention may also be used as desizing agent compositions for fibers by incorporating an enzyme such as pullulanase, isoamylase or neopullulanase.

#### EXAMPLES



Determination of amylase activity and protein content:

The amylase activity and protein content of each enzyme was determined in accordance with the following respective methods.

- 5           The determination of amylase activity was conducted by the 3,5-dinitrosalicylic acid method (DNS method). After a reaction was conducted at 50°C for 15 minutes in a reaction mixture with soluble starch contained in a 50 mM glycine buffer (pH: 10), reducing sugar formed was
- 10       determined by the DNS method. With respect to the enzymatic activity, the amount of the enzyme, which forms reducing sugar corresponding to 1  $\mu$ mol of glucose for 1 minute, was defined as 1 unit.

- The protein content was determined by means of a
- 15       Protein Assay Kit produced by Bio-Rad Laboratories making use of bovine serum albumin as a standard.

Referential Example 1:

Screening of liquefying alkaline amylase:

- Soil (about 0.5 g) was suspended in sterilized water
- 20       and subjected to a heat treatment at 80°C for 15 minutes. A supernatant of the heat-treated suspension was suitably diluted with sterilized water, and the resultant dilute solution was coated on an agar medium (Medium A) for separation. Culture was then conducted at 30°C for 2 days
- 25       to form colonies. Those on the peripheries of which transparent halo based on amylolysis had been formed were screened, and isolated as amylase-producing bacteria.

Further, the thus-isolated bacteria were inoculated on Medium B and subjected to aerobic shaking culture at 30°C for 2 days. After the culture, the resistance performance to a chelating agent (EDTA) of a supernatant centrifugally separated was determined, and its optimum pH was further measured to screen the liquefying alkaline  $\alpha$ -amylase-producing bacteria.

*Bacillus* sp. KSM-K38 (FERM BP-6946) and *Bacillus* sp. KSM-K36 (FERM BP-6945) strains were able to be obtained by the above-described process.

15	Medium A:	Trypton	1.5%
		Soyton	0.5%
		Sodium chloride	0.5%
		Colored starch	0.5%
		Agar	1.5%
20		Na <sub>2</sub> CO <sub>3</sub>	0.5%
		(pH 10)	
	Medium B:	Trypton	1.5%
		Soyton	0.5%
		Sodium chloride	0.5%
		Soluble starch	1.0%
		Na <sub>2</sub> CO <sub>3</sub>	0.5%
		(pH 10)	

The mycological natures of the KSM-K38 and KSM-K36 strains are shown in Table 1.

Table 1

	KSM-K36 strain	KSM-K38 strain
(a) Results of microscopic observation	Bacilli having sizes of 1.0-1.2 $\mu\text{m}$ x 2.4-5.4 $\mu\text{m}$ for K36 stain and 1.0-1.2 $\mu\text{m}$ x 1.8-3.8 $\mu\text{m}$ for K38 strain. Oval endospores (1.0-1.2 $\mu\text{m}$ x 1.2-1.4 $\mu\text{m}$ ) are formed at near end or the center thereof. Having periplasmic flagella and motility. Gram staining is positive. Having no acid-fast.	
(b) Growth state on various media: Incidentally, the strains are alkaliphilic and so 0.5% sodium carbonate was added to media used in the following tests.		
•Nutrient agar plate culture	Good growth state. Form of colonies is circular. Smooth surface and rough periphery. Color of colonies is pale-ocher.	Good growth state. Form of colonies is circular. Smooth surface and smooth periphery. Color of colonies is yellowish-brown.
•Nutrient agar slant culture	Grown.	Grown.
•Nutrient broth liquid culture	Grown.	Grown.
•Nutrient broth gelatin stab culture	Good growth state. No gelatin liquefaction is observed.	Good growth state. No gelatin liquefaction is observed.
•Litmus milk medium	Not changed.	Not changed.
(c) Physiological nature:		
•Reduction of nitrate and denitrification	Reduction of nitrate is positive. Denitrification is negative.	Reduction of nitrate is positive. Denitrification is negative.
•MR test	Failed to judge because the medium is alkaline.	Failed to judge because the medium is alkaline.
•V-P test	Negative.	Negative.
•Formation of indole	Negative.	Negative.
•Formation of hydrogen sulfide	Negative.	Negative.
•Hydrolysis of starch	Negative.	Negative.
•Citrate utilization	Grown on Christensen medium but not grown on Coker and Simmons media.	Grown on Christensen medium but not grown on Coker and Simmons media.
•Utilization of inorganic nitrogen source	Nitrate is utilized, but ammonium salt is not utilized.	Nitrate is utilized, but ammonium salt is not utilized.
•Formation of pigment	Formation of pale-yellow pigment on King B medium.	Negative.
•Urease	Negative	Negative
•Oxidase	Negative	Negative
•Catalase	Negative	Negative
•Range of growth	Temperature range for growth is 15-40°C, and optimum temperature range for growth is 30-37°C. pH range for growth is pH 8.0-11.0, and optimum pH for growth is pH 10.0-11.0.	Temperature range for growth is 15-40°C, and optimum temperature range for growth is 30°C. pH range for growth is pH 9.0-11.0, and optimum pH for growth is the same as described above.
•Behavior against oxygen	Aerobic.	Aerobic.
•O-F test	Not grown.	Not grown.
•Sugar utilization	D-galactose, D-xylose, L-arabinose, lactose, glycerol, melibiose, ribose, D-glucose, D-mannose, maltose, sucrose, trehalose, D-mannit, starch, raffinose and D-fructose are utilized.	D-galactose, D-xylose, L-arabinose, lactose, glycerol, melibiose, ribose, D-glucose, D-mannose, maltose, sucrose, trehalose, D-mannit, starch, raffinose and D-fructose are utilized.
•Growth on salt-containing medium	Grown at a salt concentration of 12%, but not grown at a concentration of 15%.	Grown at a salt concentration of 12%, but not grown at a concentration of 15%.

Reference Example 2:

Culture of KSM-K38 and KSM-K36 strains:

The KSM-K38 or KSM-K36 strain was inoculated on the liquid medium B used in Referential Example 1 to conduct shaking culture at 30°C for 2 days. The amylase activity (at pH 8.5) of a supernatant centrifugally separated was determined. As a result, these strains had activities of 557 U and 1177 U per liter of the medium, respectively.

Referential Example 3:

10 Purification of liquefying alkaline amylase:

Ammonium sulfate was added to the resultant culture supernatant of the KSM-38 strain obtained in Referential Example 2 to 80% saturation. After stirring the resultant mixture, precipitate formed was collected and dissolved in 10 mM Tris-hydrochloride buffer (pH: 7.5) containing 2 mM  $\text{CaCl}_2$  and dialyzed overnight against the same buffer. The dialyzate thus obtained was passed through a DEAE-Toyopearl 650M column equilibrated with the same buffer and caused to be adsorbed on the column, and the intended enzyme was eluted with the same buffer by 0-1 M gradient of sodium chloride concentration. After the active fraction was dialyzed against the same buffer, an active fraction obtained by gel filtration column chromatography was dialyzed against the above-described buffer, thereby obtaining a purified enzyme which gave a single band on both polyacrylamide gel electrophoresis (gel concentration: 10%) and sodium dodecyl sulfate (SDS)

electrophoresis. Incidentally, a purified enzyme was also able to be obtained from the culture supernatant of the KSM-K36 strain in accordance with the same process as described above.

5 Reference Example 4:

Properties of enzyme:

(1) Action:

Both enzymes decompose the  $\alpha$ -1,4-glycoside bonds of starch, amylose, amylopectin and partially decomposed  
10 products thereof and produce glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7) from amylose.

However, the enzymes do not act on pullulan.

(2) pH stability (Britton-Robinson's buffer):

15 Both enzymes exhibit a residual activity of at least 70% in a pH range of 6.5 to 11 under treatment conditions of 40°C and 30 minutes.

(3) Action temperature range and optimum action temperature:

20 Both enzymes act in a wide temperature range of 20 to 80°C and have an optimum action temperature of 50 to 60°C.

(4) Temperature stability:

Enzyme was incubated in a 50 mM glycine-sodium  
25 hydroxide buffer (pH: 10) at various temperature for 30 minutes and then residual enzymatic activity was measured. As a result, both enzymes showed a residual activity of at

least 80% at 40°C and a residual activity of about 60% even at 45°C.

(5) Molecular weight:

Both enzymes have a molecular weight of 55,000 ± 5,000 as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(6) Isoelectric point:

Both enzymes have an isoelectric point of about 4.2 as measured by isoelectric focusing.

10 (7) Influence of surfactant:

Even when both enzymes are treated at pH 10 and 30°C for 30 minutes in a 0.1% solution of each of various surfactants such as sodium linear alkylbenzenesulfonates, sodium alkylsulfates, sodium polyoxyethylene alkylsulfates, 15 sodium α-olefinsulfonates, the sodium salts of α-sulfonated fatty acid esters, sodium alkylsulfonates, SDS, soap and Softanol, they scarcely undergo inhibition of their activities (residual activity: at least 90%).

(8) Influence of metal salt:

20 Both enzymes were treated at pH 10 and 30°C for 30 minutes with each of various metal salts, thereby determining the influence thereof.

The K38 strain is inhibited by 1 mM  $Mn^{2+}$  (inhibitory rate: about 75%) and somewhat inhibited by both 1 mM  $Sr^{2+}$  25 and  $Cd^{2+}$  (inhibitory rate: 30 to 40%).

Example 1: Cloning of liquefying α-amylase gene

A chromosome DNA extracted from cells of the KSM-K38

strain in accordance with the method by Saito & Miura  
(Biochim. Biophys. Acta, 72, 619, 1961) was used as a  
template to amplify a gene fragment (about 1.5 kb)  
encoding a liquefying alkaline  $\alpha$ -amylase (hereinafter  
5 referred to as "K38AMY") having an amino acid sequence set  
forth in SEQ ID NO:1 by PCR making use of primers K38US  
(SEQ ID NO: 19) and K38DH (SEQ ID NO: 20). The thus-  
amplified fragment was cleaved with a restriction enzyme  
*Sal* I, and then inserted into a *Sal* I-*Sma* I site of an  
10 expression vector pHSP64 (Japanese Patent Application  
Laid-Open No. 217781/1994), thereby preparing a  
recombinant plasmid pHSP-K38 with a structural gene of  
K38AMY bonded to a trailing end of a potent promoter  
derived from the alkaline cellulase gene of a *Bacillus* sp.  
15 KSM-64 (FERM P-10482) strain contained in pHSP64 (Fig. 1).

Similarly, a gene fragment (about 1.5 kb) encoding a  
liquefying alkaline  $\alpha$ -amylase (hereinafter referred to as  
"LAMY") having an amino acid sequence set forth in SEQ ID  
NO:2, which had been obtained by using a chromosome DNA  
20 extracted from cells of a *Bacillus* sp. KSM-AP1378 (FERM  
BP-3048) strain (Japanese Patent Application Laid-Open No.  
336392/1998) as a template, and amplified by PCR making  
use of primers LAUS (SEQ ID NO: 21) and LADH (SEQ ID NO:  
22) was inserted into a *Sal* I-*Sma* I site of an expression  
25 vector pHSP64 in the same manner as described above,  
thereby preparing a recombinant plasmid pHSP-LAMY (Fig. 1).  
Example 2: Preparation of mutant K38AMY gene-1

A Site-Directed Mutagenesis System Mutan-Super Express Km Kit produced by Takara Shuzo Co., Ltd. was used for a site-specific mutation. The recombinant plasmid pHSP-K38 obtained in Example 1 was first used as a  
5 template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20), thereby amplifying a fragment of about 2.1 kb from the leading end of a potent promoter derived from the KSM-64 strain to the trailing end of the liquefying alkaline  $\alpha$ -amylase gene. This  
10 fragment was inserted into a *Sma* I site of a plasmid vector pKF19k attached to the above kit to prepare a recombinant plasmid pKF19-K38 for introduction of mutation (Fig. 2).

After various kinds of oligonucleotide primers for  
15 introduction of site-specific mutation respectively set forth in SEQ ID NO:6 to NO:15 were 5'-phosphorylated with a T4 DNA kinase, each of the resultant products and pKF19-K38 were used to conduct a mutation-introducing reaction in accordance with a method described in the kit, and an  
20 *Escherichia coli* MV1184 strain (Competent Cell MV1184, product of Takara Shuzo Co., Ltd.) was transformed with the resultant reaction product. Recombinant plasmids were extracted from the resultant transformants to conduct base sequence analysis, thereby confirming the mutation.

25 The mutation-introduced gene was made a template plasmid upon introduction of a different mutation by inserting an expression promoter region and a mutant



K38AMY gene portion into the *Sma* I site of pKF19k again, thereby introducing another mutation in accordance with the same process as described above.

Each of the thus-obtained mutant recombinant  
5 plasmids was used as a template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20), thereby amplifying each of mutant K38AMY gene fragments. This fragment was cleaved with a *Sal* I and then inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64  
10 (Japanese Patent Application Laid-Open No. 217781/1994) to prepare a plasmid for production of mutant K38AMY (Fig. 1).  
Example 3: Preparation of mutant K38AMY gene-2 (chimera with LAMY gene)

Recombinant PCR was used for a mutation in which the  
15 N-terminal region of the K38AMY gene is replaced by its corresponding region of an LAMY gene (Fig. 3). The recombinant plasmid pHSP-K38 obtained in Example 1 was first used as a template to conduct PCR making use of primers K38DH (SEQ ID NO: 20) and LA-K38 (SEQ ID NO: 17),  
20 thereby amplifying a fragment encoding a sequence from the 20th Gln to C-terminal of the amino acid sequence of K38AMY set forth in SEQ ID NO: 1. On the other hand, the recombinant plasmid pHSP-LAMY was used as a template to conduct PCR making use of primers CLUBG (SEQ ID NO: 23)  
25 and LA-K38R (SEQ ID NO: 18), thereby amplifying a gene fragment encoding a sequence from the leading end of the potent promoter to the 21st Gly of the amino acid sequence

of LAMY set forth in SEQ ID NO: 2. Second PCR making use of both DMA fragments, and primers CLUBG (SEQ ID NO: 23) and K38DH (SEQ ID NO: 20) was conducted, thereby amplifying a gene fragment (about 2.1 kb) encoding a substituted mutant enzyme (hereinafter abbreviated as "LA-K38AMY") in which both fragments having respective complementary sequences derived from the primers LA-K38 (SEQ ID NO: 17) and LA-K38R (SEQ ID NO: 18) were bonded to the terminal, and a region encoding a sequence from the 1st His to the 21st Gly of LAMY and successively a region encoding a sequence from the 20th Gln to the C-terminal of K38AMY were bonded to the trailing end of the potent promoter. This gene fragment was cleaved with *Sal* I and inserted into a *Sal* I-*Sma* I site of an expression vector pHSP64 (Japanese Patent Application Laid-Open No. 217781/1994), thereby preparing a plasmid for production of mutant K38AMY (Fig. 1).

Example 4: Production of mutant liquefying alkaline  $\alpha$ -amylase

Each of the various plasmids for production of mutant K38AMY obtained in Examples 2 and 3 was introduced into a *Bacillus subtilis* ISW 1214 strain (*leuA metB5 hsdM1*) in accordance with the protoplast method (Mol. Gen. Genet., 168, 111, 1979) to culture the resultant recombinant *Bacillus subtilis* at 30°C for 3 days in a liquid medium (containing 8% of corn steep liquor; 1% of meat extract; 0.02% of potassium primary phosphate; 5% of

maltose; 5 mM of calcium chloride; and 15 µg/mL of tetracycline). The resultant culture supernatant was dialyzed against a Tris-HCl buffer (pH: 7.0), and the dialyzate was caused to be adsorbed on a DEAE-Toyopearl 5 650M column equilibrated with the same buffer, and eluted by gradient of NaCl concentration. This eluate was dialyzed against a 10 mM glycine buffer (pH: 10.0), thereby obtaining a purified enzyme of each mutant K38AMY. Example 5: Assay of heat resistance-1

10 Purified preparations of an enzyme (abbreviated as "Y11F") with the 11th Tyr in SEQ ID NO:1 replaced by Phe, an enzyme (abbreviated as "N49S") with the 49th Asn replaced by Ser, an enzyme (abbreviated as "E84Q") with the 84th Glu replaced by Gln, an enzyme (abbreviated as 15 "S144P") with the 144th Ser replaced by Pro, an enzyme (abbreviated as "Q167E") with the 167th Gln replaced by Glu, an enzyme (abbreviated as "Y169K") with the 169th Tyr replaced by Lys, an enzyme (abbreviated as "A178Q") with the 178th Ala replaced by Gln, an enzyme (abbreviated as 20 "E188D") with the 188th Glu replaced by Asp, an enzyme (abbreviated as "N190F") with the 190th Asn replaced by Phe, and an enzyme (abbreviated as "Q209V") with the 209th Gln replaced by Val were obtained in accordance with the processes described in Examples 1, 2 and 4, and their heat 25 resistance was assayed by the following method. As a control, wild type K38AMY was used.

Each enzyme was added to a 50 mM glycine buffer (pH:

10.0) preincubated at 50°C so as to give a concentration of about 1.2 U/mL, and after 30 minutes, the buffer was sampled to determine the residual amylase activity of the enzyme in accordance with the method described above in

5 EXAMPLES. The activity of the enzyme at the start is regarded as 100% to determine a relative activity, thereby regarding it as the residual amylase activity. The results are shown in Table 2. In the wild type K38AMY, the residual activity was decreased to 15%, while all the

10 mutant enzymes exhibited a high residual activity compared with the wild type.

Table 2

Enzyme	Residual activity (%) after 30 minutes
Wild type	15
Y11F	40
N49S	30
E84Q	25
S144P	30
Q167E	46
Y169K	63
A178Q	20
E188D	30
N190F	70
Q209V	40

Example 6: Assay of heat resistance-2

15 Mutant enzymes with Q167E, Y169K, N190F and Q209V among the mutations described in Example 5 combined in the following manner were prepared in accordance with the processes described in Examples 1, 2 and 4.

Q167E/Y169K (abbreviated as "QEYK", prepared by  
using primer of SEQ ID NO: 16)

N190F/Q209V (abbreviated as "NFQV")

Q167E/Y169K/N190F/Q209V (abbreviated as "QEYK/NFQV")

5 With respect to these enzymes, the heat resistance  
was assayed by a method similar to Example 5. However, the  
temperature in the heat treatment was changed to 55°C, and  
Q167E, Y169K, N190F and Q209V were used as controls. As a  
result, as shown in Table 3, all the mutants were observed  
10 being improved in heat resistance by the combination, and  
QEYK/NFQV obtained by combining 4 mutations exhibited a  
residual activity of 85% after 30 minutes even at 55°C.

Table 3

Enzyme	Residual activity (%) after 30 minutes
Q167E	7
Y169K	14
QEYK	45
N190F	20
Q209V	1
NFQV	40
QEYK/NFQV	85

15 Example 7: Assay of heat resistance-3

The following mutant enzymes with the mutation NFQV  
described in Example 6 combined with S144P described in  
Example 5, and further combined with a mutation of  
replacement of 16th Gln by Pro (abbreviated as "E16P")  
20 were prepared in accordance with the processes described

in Examples 1, 2 and 4.

S144P/NFQV (abbreviated as "SP/NFQV")

E16P/S144P/NFQV (abbreviated as "EPSP/NFQV")

With respect to these enzymes, the heat resistance  
5 was assayed by a method (50°C) similar to Example 5. As a  
result, as shown in Table 4, improvement in heat  
resistance was observed by combining E16P with SP/NFQV.

Table 4

Enzyme	Residual activity (%) after 30 minutes
SP/NFQV	40
EPSP/NFQV	50

10 Example 8: Assay of heat resistance-4

The following mutant enzymes with QEYK/NFQV among  
the mutations described in Example 6 suitably combined  
with a mutation (abbreviated as "M107L") with the 107th  
Met in SEQ ID NO:1 replaced by Leu, a mutation  
15 (abbreviated as "H205R") with the 205th His replaced by  
Arg, and N49S among the mutations described in Example 5  
were prepared in accordance with the processes described  
in Examples 1, 2 and 4.

M107L/QEYK/NFQV (abbreviated as "ML/QEYK/NFQV")

20 N49S/M107L/QEYK/NFQV (abbreviated as  
"NSML/QEYK/NFQV")

N49S/M107L/H205R/QEYK/NFQV (abbreviated as  
"NSMLHR/QEYK/NFQV")

With respect to these enzymes, the heat resistance was assayed by a method similar to Example 5. However, the temperature in the heat treatment was changed to 60°C.

As a result, heat resistance was additionally improved by combining ML/QEYK/NFQV with N49S, further H205R, and NSMLHR/QEYK/NFQV exhibited a residual activity of 75% after 30 minutes even at 60°C (Table 5)

Table 5

Enzyme	Residual activity (%) after 30 minutes
ML/QEYK/NFQV	30
NSML/QEYK/NFQV	50
NSMLHR/QEYK/NFQV	75

#### 10 Example 9: Assay of heat resistance-5

A mutant enzyme LA-K38AMY with a sequence from the 1st Asp to the 19th Gly of K38AMY replaced by a sequence from the 1st His to the 21st Gly of LAMY was obtained in accordance with the processes described in Examples 1, 3 and 4. The heat resistance of this enzyme was assayed by the method described in Example 5. As a result, as shown in Table 6, improvement in heat resistance by the replacement was observed.

Table 6

Enzyme	Residual activity (%) after 30 minutes
Wild type	15
LA-K38AMY	33

Example 10: Assay of heat resistance-6

Into the gene of the mutant enzyme QEYK/NFQV described in Example 6, was introduced a mutation with a sequence from the 1st Asp to the 19th Gly replaced by a  
5 sequence from the 1st His to the 21st Gly of LAMY in accordance with the same processes as in Examples 1 and 3. With respect to a mutant enzyme LA-K38AMY/QEYK/NFQV obtained by using this enzyme in accordance with the process described in Example 4, the heat resistance was  
10 assayed by the same method (heat treatment temperature: 60°C) as in Example 8.

As a result, heat resistance was additionally improved by the combination, and LA-K38AMY/QEYK/NFQV exhibited a residual activity of 63% after 30 minutes even  
15 at 60°C (Table 7)

Table 7

Enzyme	Residual activity (%) after 30 minutes
LA-K38AMY	1
QEYK/NFQV	40
LA-K38AMY/QEYK/NFQV	63

Example 11: Detergent composition for automatic dish washer

20 A detergent composition for automatic dish washer was produced in accordance with a formulation shown in Table 8, and various mutant enzymes were separately incorporated into this detergent composition to conduct a



washing test. As a result, the mutant enzymes exhibited an excellent detergent effect compared with the wild type enzyme when the enzymes having the same activity value as each other are added.

5 Table 8

Composition of detergent	(%)
Pluronic L-61	2.2
Sodium carbonate	24.7
Sodium hydrogencarbonate	24.7
Sodium percarbonate	10.0
Sodium silicate No. 1	12.0
Trisodium citrate	20.0
Polypropylene glycol	2.2
Silicone KST-04 (product of Toshiba silicone Co., Ltd.)	0.2
Socarane CP-A45 (product of BASF AG)	4.0

The mutant  $\alpha$ -amylases according to the present invention have excellent properties of high resistance to chelating agents, high specific activity in an alkaline region, excellent stability to heat, and are hence useful for detergents for automatic dish washer, laundry detergents, compositions for liquefaction and saccharification of starch, and desizing agents for fibers.

SEQUENCE LISTING

<110>KAO CORPORATION

<120>New mutant alpha-amylase

<130>

<150>JP P1999-163569

<151>1999-06-10

<160>23

<210>1

<211>480

<212>PRT

<213>Bacillus sp. KSM-K38

<400>1

Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu

5

10

15

Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu

20

25

30

Ser Asp Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly

35

40

45

Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu

50	55	60
Gly Glu Phe Asn Gln Lys	Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys	
65	70	75
Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn		80
	85	90
Val Tyr Gly Asp Val Val Met Asn His Lys Met Gly Ala Asp Phe Thr		95
100	105	110
Glu Ala Val Gln Ala Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp		
115	120	125
Ile Ser Gly Ala Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser		
130	135	140
Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe		
145	150	155
Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg		160
	165	170
Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn		175
180	185	190
Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val		
195	200	205
Gln Asp Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp		
210	215	220
Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr		
225	230	235
Thr Ser Asp Trp Val Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu		240
	245	250
Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe		255

260	265	270	
Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu			
275	280	285	
Asn Tyr Asn Phe Tyr Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met			
290	295	300	
Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Met His Ala			
305	310	315	320
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu			
325	330	335	
Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu			
340	345	350	
Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly			
355	360	365	
Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu			
370	375	380	
Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe			
385	390	395	400
Asp His Trp Asp Val Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg			
405	410	415	
Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser			
420	425	430	
Lys Trp Met Tyr Val Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp			
435	440	445	
Leu Thr Gly Asn Asn Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp			
450	455	460	
Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln			

465

470

475

480

&lt;210&gt;2

&lt;211&gt;485

&lt;212&gt;PRT

&lt;213&gt;Bacillus sp. KSM-AP1378

&lt;400&gt;2

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His

5

10

15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala

20

25

30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp

35

40

45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr

50

55

60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly

65

70

75

80

Thr Arg Ser Gln Leu Gln Gly Ala Val Thr Ser Leu Lys Asn Asn Gly

85

90

95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp

100

105

110

Gly Thr Glu Met Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn

115

120

125

Gln Glu Ile Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp

130

135

140

Phe	Pro	Gly	Arg	Gly	Asn	Thr	His	Ser	Asn	Phe	Lys	Trp	Arg	Trp	Tyr
145					150					155					160
His	Phe	Asp	Gly	Thr	Asp	Trp	Asp	Gln	Ser	Arg	Gln	Leu	Gln	Asn	Lys
				165				170						175	
Ile	Tyr	Lys	Phe	Arg	Gly	Thr	Gly	Lys	Ala	Trp	Asp	Trp	Glu	Val	Asp
				180				185						190	
Ile	Glu	Asn	Gly	Asn	Tyr	Asp	Tyr	Leu	Met	Tyr	Ala	Asp	Ile	Asp	Met
		195					200					205			
Asp	His	Pro	Glu	Val	Ile	Asn	Glu	Leu	Arg	Asn	Trp	Gly	Val	Trp	Tyr
		210				215					220				
Thr	Asn	Thr	Leu	Asn	Leu	Asp	Gly	Phe	Arg	Ile	Asp	Ala	Val	Lys	His
225				230						235					240
Ile	Lys	Tyr	Ser	Tyr	Thr	Arg	Asp	Trp	Leu	Thr	His	Val	Arg	Asn	Thr
				245				250					255		
Thr	Gly	Lys	Pro	Met	Phe	Ala	Val	Ala	Glu	Phe	Trp	Lys	Asn	Asp	Leu
			260					265					270		
Ala	Ala	Ile	Glu	Asn	Tyr	Leu	Asn	Lys	Thr	Ser	Trp	Asn	His	Ser	Val
		275					280					285			
Phe	Asp	Val	Pro	Leu	His	Tyr	Asn	Leu	Tyr	Asn	Ala	Ser	Asn	Ser	Gly
		290					295				300				
Gly	Tyr	Phe	Asp	Met	Arg	Asn	Ile	Leu	Asn	Gly	Ser	Val	Val	Gln	Lys
305				310						315					320
His	Pro	Ile	His	Ala	Val	Thr	Phe	Val	Asp	Asn	His	Asp	Ser	Gln	Pro
				325						330				335	
Gly	Glu	Ala	Leu	Glu	Ser	Phe	Val	Gln	Ser	Trp	Phe	Lys	Pro	Leu	Ala
			340					345					350		

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr  
 355 360 365  
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ser Met Lys Ser  
 370 375 380  
 Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Tyr Ala Tyr Gly Thr  
 385 390 395 400  
 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu  
 405 410 415  
 Gly Asp Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp  
 420 425 430  
 Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys His Lys Ala Gly  
 435 440 445  
 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Ser Gly Thr Val Thr Ile  
 450 455 460  
 Asn Ala Asp Gly Trp Gly Asn Phe Thr Val Asn Gly Gly Ala Val Ser  
 465 470 475 480  
 Val Trp Val Lys Gln  
 485

<210>3

<211>1753

<212>DNA

<213>Bacillus sp. KSM-K38

<220>

<221>sig\_peptide

<222>(162)..(224)

<220>

<221>mat\_peptide

<222>(225)..(1664)

<220>

<221>CDS

<222>(162)..(1664)

<400>3

```
gtatgcgaaa cgaigcgcaa aactgcgcaa ctactagcac tcitcaggga ctaaaccacc 60
ttttttccaa aaatgacatc atataaacia atttgtctac caatcactat ttaaagcigt 120
ttaigatata tgtaagcggt atcattaaaa ggaggtatit g atg aga aga tgg gta 176
gta gca atg ttg gca gtg tta ttt tta ttt cct tcg gta gta gtt gca 224
gat gga ttg aac ggt acg atg atg cag tat tat gag tgg cat ttg gaa 272
Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu
1          5          10          15
aac gac ggg cag cat tgg aat cgg ttg cac gat gat gcc gca gct ttg 320
Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu
20          25          30
agt gat gct ggt att aca gct att tgg att ccg cca gcc tac aaa ggt 368
Ser Asp Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly
35          40          45
aat agt cag gcg gat gtt ggg tac ggt gca tac gat ctt tat gat tta 416
Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
```



50	55	60	
gga gag ttc aat caa aag ggt act gtt cga acg aaa tac gga act aag	464		
Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys			
65	70	75	80
gca cag ctt gaa cga gct att ggg tcc ctt aaa tct aat gat atc aat	512		
Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn			
85	90	95	
gta tac gga gat gtc gtg atg aat cat aaa atg gga gct gat ttt acg	560		
Val Tyr Gly Asp Val Val Met Asn His Lys Met Gly Ala Asp Phe Thr			
100	105	110	
gag gca gtg caa gct gtt caa gta aat cca acg aat cgt tgg cag gat	608		
Glu Ala Val Gln Ala Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp			
115	120	125	
att tca ggt gcc tac acg att gat gcg tgg acg ggt ttc gac ttt tca	656		
Ile Ser Gly Ala Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser			
130	135	140	
ggg cgt aac aac gcc tat tca gat ttt aag tgg aga tgg ttc cat ttt	704		
Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe			
145	150	155	160
aat ggt gtt gac tgg gat cag cgc tat caa gaa aat cat att ttc cgc	752		
Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg			
165	170	175	
ttt gca aat acg aac tgg aac tgg cga gtg gat gaa gag aac ggt aat	800		
Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn			
180	185	190	
tat gat tac ctg tta gga tcg aat atc gac ttt agt cat cca gaa gta	848		

Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val	
195	200
205	
caa gat gag ttg aag gat tgg ggt agc tgg ttt acc gat gag tta gat	896
Gln Asp Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp	
210	215
220	
ttg gat ggt tat cgt tta gat gct att aaa cat att cca ttc tgg tat	944
Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr	
225	230
235	240
aca tct gat tgg gtt cgg cat cag cgc aac gaa gca gat caa gat tta	992
Thr Ser Asp Trp Val Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu	
245	250
255	
ttt gtc gta ggg gaa tat tgg aag gat gac gta ggt gct ctc gaa ttt	1040
Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe	
260	265
270	
tat tta gat gaa atg aat tgg gag atg tct cta ttc gat gtt cca ctt	1088
Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu	
275	280
285	
aat tat aat ttt tac cgg gct tca caa caa ggt gga agc tat gat atg	1136
Asn Tyr Asn Phe Tyr Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met	
290	295
300	
cgt aat att tta cga gga tct tta gta gaa gcg cat ccg atg cat gca	1184
Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Met His Ala	
305	310
315	320
gtt acg ttt gtt gat aat cat gat act cag cca ggg gag tca tta gag	1232
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu	
325	330
335	

tca tgg gtt gct gat tgg ttt aag cca ctt gct tat gcg aca att ttg	1280
Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu	
340 345 350	
acg cgt gaa ggt ggt tat cca aat gta ttt tac ggt gat tac tat ggg	1328
Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly	
355 360 365	
att cct aac gat aac att tca gct aaa aaa gat atg att gat gag ctg	1376
Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu	
370 375 380	
ctt gat gca cgt caa aat tac gca tat ggc acg cag cat gac tat ttt	1424
Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe	
385 390 395 400	
gat cat tgg gat gtt gta gga tgg act agg gaa gga tct tcc tcc aga	1472
Asp His Trp Asp Val Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg	
405 410 415	
cct aat tca ggc ctt gcg act att atg tcg aat gga cct ggt ggt tcc	1520
Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser	
420 425 430	
aag tgg atg tat gta gga cgt cag aat gca gga caa aca tgg aca gat	1568
Lys Trp Met Tyr Val Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp	
435 440 445	
tta act ggt aat aac gga gcg tcc gtt aca att aat ggc gat gga tgg	1616
Leu Thr Gly Asn Asn Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp	
450 455 460	
ggc gaa ttc ttt acg aat gga gga tct gta tcc gtg tac gtg aac caa	1664

Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln  
 465 470 475 480  
 taacaaaaag ccttgagaag ggattcctcc ctaactcaag gctttcttta tgtcgcttag 1724  
 cttaacgcctt ctacgacttt gaagcttta 1753

<210>4

<211>480

<212>PRT

<213>Bacillus sp. KSM-K36

<400>4

Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu  
                           5                          10                          15  
 Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Glu Ala Leu  
                           20                          25                          30  
 Ser Asn Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly  
                           35                          40                          45  
 Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu  
                           50                          55                          60  
 Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys  
                           65                          70                          75                          80  
 Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn  
                           85                          90                          95  
 Val Tyr Gly Asp Val Val Met Asn His Lys Leu Gly Ala Asp Phe Thr  
                           100                          105                          110  
 Glu Ala Val Gln Ala Val Gln Val Asn Pro Ser Asn Arg Trp Gln Asp

115	120	125
Ile Ser Gly Val Tyr Thr	Ile Asp Ala Trp Thr Gly Phe Asp Phe Pro	
130	135	140
Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe		
145	150	155
Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Leu Phe Arg		
165	170	175
Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn		
180	185	190
Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val		
195	200	205
Gln Glu Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp		
210	215	220
Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr		
225	230	235
Thr Ser Asp Trp Val Arg His Gln Arg Ser Glu Ala Asp Gln Asp Leu		
245	250	255
Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe		
260	265	270
Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu		
275	280	285
Asn Tyr Asn Phe Tyr Arg Ala Ser Lys Gln Gly Gly Ser Tyr Asp Met		
290	295	300
Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Ile His Ala		
305	310	315
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu		

	325		330		335
Ser	Trp	Val	Ala	Asp	Trp
Phe	Lys	Pro	Leu	Ala	Tyr
Ala	Thr	Ile	Leu		
	340		345		350
Thr	Arg	Glu	Gly	Gly	Tyr
Pro	Asn	Val	Phe	Tyr	Gly
Asp	Tyr	Tyr	Gly		
	355		360		365
Ile	Pro	Asn	Asp	Asn	Ile
Ser	Ala	Lys	Lys	Asp	Met
Ile	Asp	Glu	Leu		
	370		375		380
Leu	Asp	Ala	Arg	Gln	Asn
Tyr	Ala	Tyr	Gly	Thr	Gln
His	Asp	Tyr	Phe		
	385		390		395
400					
Asp	His	Trp	Asp	Ile	Val
Gly	Trp	Thr	Arg	Glu	Gly
Thr	Ser	Ser	Arg		
	405		410		415
Pro	Asn	Ser	Gly	Leu	Ala
Thr	Ile	Met	Ser	Asn	Gly
Pro	Gly	Gly	Ser		
	420		425		430
Lys	Trp	Met	Tyr	Val	Gly
Gln	Gln	His	Ala	Gly	Gln
Thr	Trp	Thr	Asp		
	435		440		445
Leu	Thr	Gly	Asn	His	Ala
Ala	Ser	Val	Thr	Ile	Asn
Gly	Asp	Gly	Trp		
	450		455		460
Gly	Glu	Phe	Phe	Thr	Asn
Gly	Ser	Val	Ser	Val	Tyr
Val	Asn	Gln			
	465		470		475
					480

<210>5

<211>1625

<212>DNA

<213>Bacillus sp. KSM-K36

<220>

<221>sig\_peptide

<222>(40)..(102)

<220>

<221>mat\_peptide

<222>(103)..(1542)

<220>

<221>CDS

<222>(40)..(1542)

<400>5

```
atgatatatg taagcggttat cattaaaagg aggtatttg atg aaa aga tgg gta      54
gta gca atg ctg gca gtg tta ttt tta ttt cct tcg gta gta gtt gca      102
gat ggc ttg aat gga acg atg atg cag tat tat gag tgg cat cta gag      150
Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu
  1              5              10              15
aat gat ggg caa cac tgg aat cgg ttg cat gat gat gcc gaa gct tta      198
Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Glu Ala Leu
          20              25              30
agt aat gcg ggt att aca gct att tgg ata ccc cca gcc tac aaa gga      246
Ser Asn Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly
          35              40              45
aat agt cag gct gat gtt ggg tat ggt gca tac gac ctt tat gat tta      294
Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
          50              55              60
```

ggg gag ttt aat caa aaa ggt acc gtt cga acg aaa tac ggg aca aag	342
Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys	
65 70 75 80	
gct cag ctt gag cga gct ata ggg tcc cta aag tcg aat gat atc aat	390
Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn	
85 90 95	
gtt tat ggg gat gtc gta atg aat cat aaa tta gga gct gat ttc acg	438
Val Tyr Gly Asp Val Val Met Asn His Lys Leu Gly Ala Asp Phe Thr	
100 105 110	
gag gca gtg caa gct gtt caa gta aat cct tcg aac cgt tgg cag gat	486
Glu Ala Val Gln Ala Val Gln Val Asn Pro Ser Asn Arg Trp Gln Asp	
115 120 125	
att tca ggt gtc tac acg att gat gca tgg acg gga ttt gac ttt cca	534
Ile Ser Gly Val Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Pro	
130 135 140	
ggg cgc aac aat gcc tat tcc gat ttt aaa tgg aga tgg ttc cat ttt	582
Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe	
145 150 155 160	
aat ggc gtt gac tgg gat caa cgc tat caa gaa aac cat ctt ttt cgc	630
Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Leu Phe Arg	
165 170 175	
ttt gca aat acg aac tgg aac tgg cga gtg gat gaa gag aat ggt aat	678
Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn	
180 185 190	
tat gac tat tta tta gga tcg aac att gac ttt agc cac cca gag gtt	726
Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val	



195	200	205	
caa gag gaa tta aag gat tgg ggg agc tgg ttt acg gat gag cta gat			774
Gln Glu Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp			
210	215	220	
tta gat ggg tat cga ttg gat gct att aag cat att cca ttc tgg tat			822
Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr			
225	230	235	240
acg tca gat tgg gtt agg cat cag cga agt gaa gca gac caa gat tta			870
Thr Ser Asp Trp Val Arg His Gln Arg Ser Glu Ala Asp Gln Asp Leu			
245	250	255	
ttt gtc gta ggg gag tat tgg aag gat gac gta ggt gct ctc gaa ttt			918
Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe			
260	265	270	
tat tta gat gaa atg aat tgg gag atg tct cta ttc gat gtt ccg ctc			966
Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu			
275	280	285	
aat tat aat ttt tac cgg gct tca aag caa ggc gga agc tat gat atg			1014
Asn Tyr Asn Phe Tyr Arg Ala Ser Lys Gln Gly Gly Ser Tyr Asp Met			
290	295	300	
cgt aat att tta cga gga tct tta gta gaa gca cat ccg att cat gca			1062
Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Ile His Ala			
305	310	315	320
gtt acg ttt gtt gat aat cat gat act cag cca gga gag tca tta gaa			1110
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu			
325	330	335	
tca tgg gtc gct gat tgg ttt aag cca ctt gct tat gcg aca atc ttg			1158

Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu	
340	345
350	
acg cgt gaa ggt ggt tat cca aat gta ttt tac ggt gac tac tat ggg	1206
Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly	
355	360
365	
att cct aac gat aac att tca gct aag aag gat atg att gat gag ttg	1254
Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu	
370	375
380	
ctt gat gca cgt caa aat tac gca tat ggc aca caa cat gac tat ttt	1302
Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe	
385	390
395	400
gat cat tgg gat atc gtt gga tgg aca aga gaa ggt aca tcc tca cgt	1350
Asp His Trp Asp Ile Val Gly Trp Thr Arg Glu Gly Thr Ser Ser Arg	
405	410
415	
cct aat tcg ggt ctt gct act att atg tcc aat ggt cct gga gga tca	1398
Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser	
420	425
430	
aaa tgg atg tac gta gga cag caa cat gca gga caa acg tgg aca gat	1446
Lys Trp Met Tyr Val Gly Gln Gln His Ala Gly Gln Thr Trp Thr Asp	
435	440
445	
tta act ggc aat cac gcg gcg tcg gtt acg att aat ggt gat ggc tgg	1494
Leu Thr Gly Asn His Ala Ala Ser Val Thr Ile Asn Gly Asp Gly Trp	
450	455
460	
ggc gaa ttc ttt aca aat gga gga tct gta tcc gtg tat gtg aac caa	1542
Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln	
465	470
475	480

taataaaaag ccttgagaag ggattccicc ctaactcaag gctttcttta tgcgittag 1602  
ctcaacgctt ctacgaagct tta 1625

<210>6

<211>30

<212>DNA

<213>Artificial Sequence

<400>6

atgatgcagt attttgagtg gcatttggaa 30

<210>7

<211>33

<212>DNA

<213>Artificial Sequence

<400>7

tatgagtggc atttgccaaa cgacgggcag cat 33

<210>8

<211>33

<212>DNA

<213>Artificial Sequence

<400>8

ccagcctaca aaggtagtag tcaggcggat gtt 33

<210>9

<211>21

<212>DNA

<213>Artificial Sequence

<400>9

gcacagcttc aacgagctat t 21

<210>10

<211>21

<212>DNA

<213>Artificial Sequence

<400>10

tttcgacttt ccagggcgta a 21

<210>11

<211>33

<212>DNA

<213>Artificial Sequence

<400>11

catattttcc gctttcaaaa tacgaactgg aac 33

<210>12

<211>33

<212>DNA

<213>Artificial Sequence

<400>12

aactggcgag tggatgatga gaacggtaat tat 33

<210>13

<211>25

<212>DNA

<213>Artificial Sequence

<400>13

tggatgaaga gticggtaat tatga 25

<210>14

<211>33

<212>DNA

<213>Artificial Sequence

<400>14

aatatcgact ttagtcgtcc agaagtacaa gat 33

<210>15

<211>33

<212>DNA

<213>Artificial Sequence

<400>15

agtcacccag aggtcgtaga tgagttgaag gat 33

<210>16

<211>33

<212>DNA

<213>Artificial Sequence

<400>16

gttgactggg atgagcgcaa acaagaaaat cat

<210>17

<211>34

<212>DNA

<213>Artificial Sequence

<400>17

atttgccaaa tgacgggcag cattggaatc gggtt 34

<210>18

<211>34

<212>DNA

<213>Artificial Sequence

<400>18

aaccgattcc aatgctgccc gtcatttggc aaat 34

<210>19

<211>40

<212>DNA

<213>Artificial Sequence

<400>19

gggtcgacca gcacaagccg atggattgaa cggtacgatg 40

<210>20

<211>29

<212>DNA

<213>Artificial Sequence

<400>20

taaagctttt gttattgggt cacgtacac 29

<210>21

<211>30

<212>DNA

<213>Artificial Sequence

<400>21

gagtcgacca gcacaagccc atcataatgg 30

<210>22

<211>21

<212>DNA

<213>Artificial Sequence

<400>22

taaagcttca atttatattg g 21

<210>23

<211>27

<212>DNA

<213>Artificial Sequence

<400>23

ccagatctac ttaccatttt agagtca 27



WHAT IS CLAIMED IS:

1. A mutant  $\alpha$ -amylase obtained by making replacement  
or deletion of at least one residue of amino acid residues  
5 respectively corresponding to the 11th Tyr, 16th Glu, 49th  
Asn, 84th Glu, 144th Ser, 167th Gln, 169th Tyr, 178th Ala,  
188th Glu, 190th Asn, 205th His and 209th Gln in the amino  
acid sequence set forth in SEQ ID NO:1 in an  $\alpha$ -amylase  
having said amino acid sequence, or an  $\alpha$ -amylase having a  
10 homology of at least 70% to said amino acid sequence.

2. A mutant  $\alpha$ -amylase obtained by making replacement  
of a sequence corresponding to 11 to 100 amino acid  
residues from the amino terminal in the amino acid  
15 sequence set forth in SEQ ID NO:1 in an  $\alpha$ -amylase having  
said amino acid sequence, or an  $\alpha$ -amylase having a  
homology of at least 70% to said amino acid sequence by an  
amino acid sequence of another liquefying  $\alpha$ -amylase  
corresponding to said sequence of the amino acid residues.

20

3. The mutant  $\alpha$ -amylase according to Claim 2,  
wherein a sequence corresponding to amino acid residues  
from the 1st Asp to the 19th Gly in the amino acid  
sequence of SEQ ID NO:1 is replaced by an amino acid  
25 sequence of another liquefying  $\alpha$ -amylase corresponding to  
said amino acid sequence.

4. The mutant  $\alpha$ -amylase according to Claim 2 or 3, wherein said another liquefying  $\alpha$ -amylase has the amino acid sequence set forth in SEQ ID NO:2.

5 5. A mutant  $\alpha$ -amylase obtained by introducing a mutation into an  $\alpha$ -amylase having the amino acid sequence set forth in SEQ ID NO:1 or an  $\alpha$ -amylase having a homology of at least 70% to said amino acid sequence with at least two kinds of replacement or deletion selected from the  
10 replacement or deletion of the amino acid residues set forth in Claim 1, and the replacement of the amino acid sequence set forth in any one of Claims 2 to 4 combined with each other.

15 6. The mutant  $\alpha$ -amylase according to Claim 5, wherein the replacement of the amino acid residue comprises replacing an amino acid residue corresponding to the 11th Tyr in the amino acid sequence of SEQ ID NO:1 by Phe, an amino acid residue corresponding to the 16th Glu  
20 by Pro, an amino acid residue corresponding to the 49th Asn by Ser, an amino acid residue corresponding to the 167th Gln by Glu, an amino acid residue corresponding to the 169th Tyr by Lys, an amino acid residue corresponding to the 190th Asn by Phe, an amino acid residue  
25 corresponding to the 205th His by Arg, or an amino acid residue corresponding to the 209th Gln by Val, and the replacement of the amino acid sequence comprises replacing

an amino acid sequence from the 1st Asp to the 19th Gly in the amino acid sequence of SEQ ID NO:1 by an amino acid sequence from the 1st His to the 21st Gly in the amino acid sequence set forth in SEQ ID NO:2.

5

7. A gene encoding the mutant  $\alpha$ -amylase according to any one of Claims 1 to 6, or a vector containing said gene.

8. Cells transformed by the vector according to  
10 Claim 7.

9. A process for producing a mutant  $\alpha$ -amylase, comprising culturing the transformed cells according to Claim 8.

15

10. A detergent composition comprising the mutant  $\alpha$ -amylase according to any one of Claims 1 to 6.

## ABSTRACT OF THE DISCLOSURE

The invention relates to a mutant  $\alpha$ -amylase obtained by making replacement or deletion of at least one of amino acid residues such as the 167th Gln, 169th Tyr and 178th Ala in the amino acid sequence set forth in SEQ ID NO:1 in an  $\alpha$ -amylase having said amino acid sequence, or an  $\alpha$ -amylase having a homology of at least 70% to said amino acid sequence, a gene encoding the mutant  $\alpha$ -amylase, a vector, transformed cells, a process for producing a mutant  $\alpha$ -amylase, comprising culturing the transformed cells, and a detergent composition comprising the mutant  $\alpha$ -amylase.

The mutant  $\alpha$ -amylase of the invention has excellent properties of high resistance to chelating agents, high specific activity in an alkaline region and excellent stability to heat, and is hence useful for detergents for automatic dish washer, laundry detergents and the like.

Fig. 1

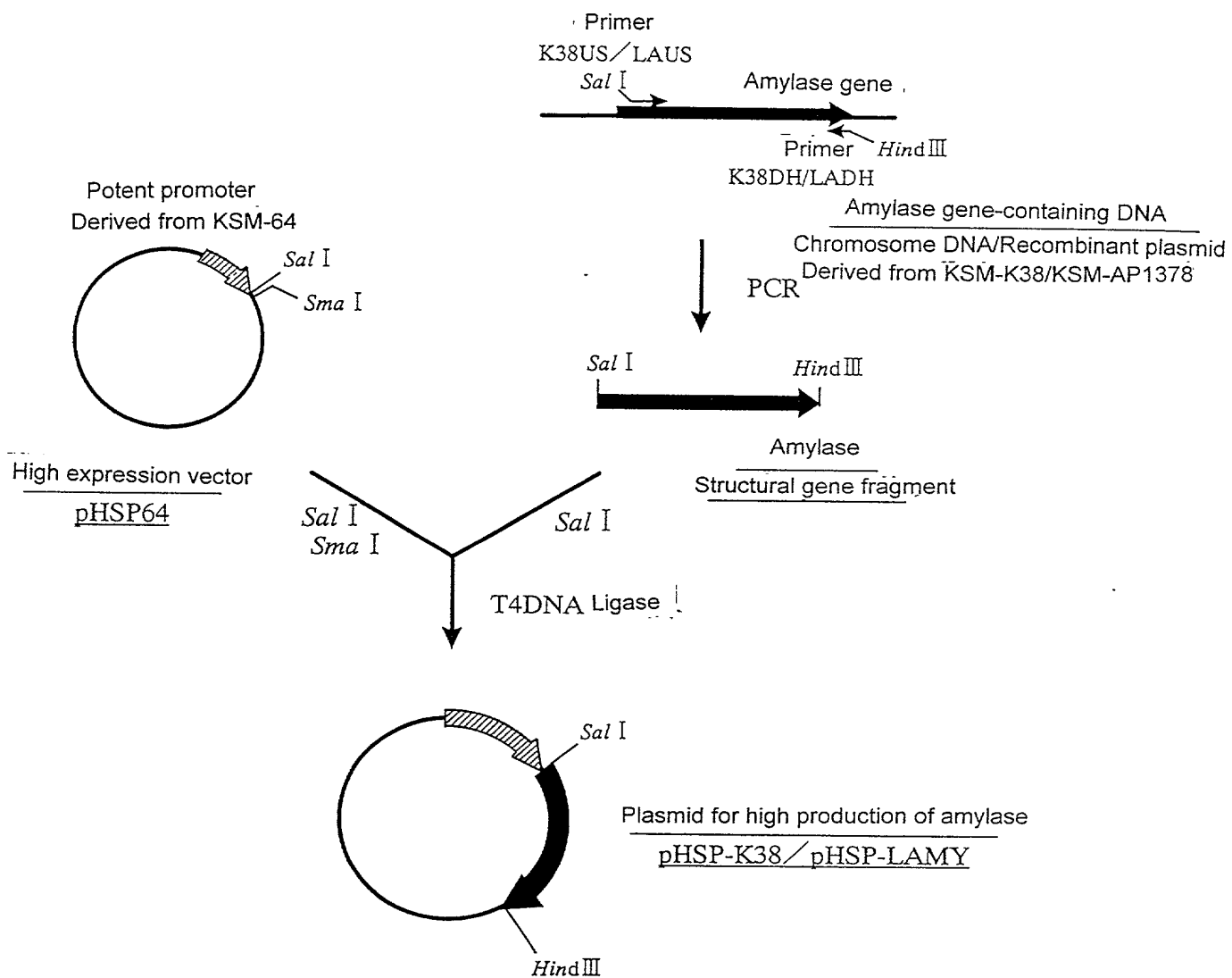


Fig. 2

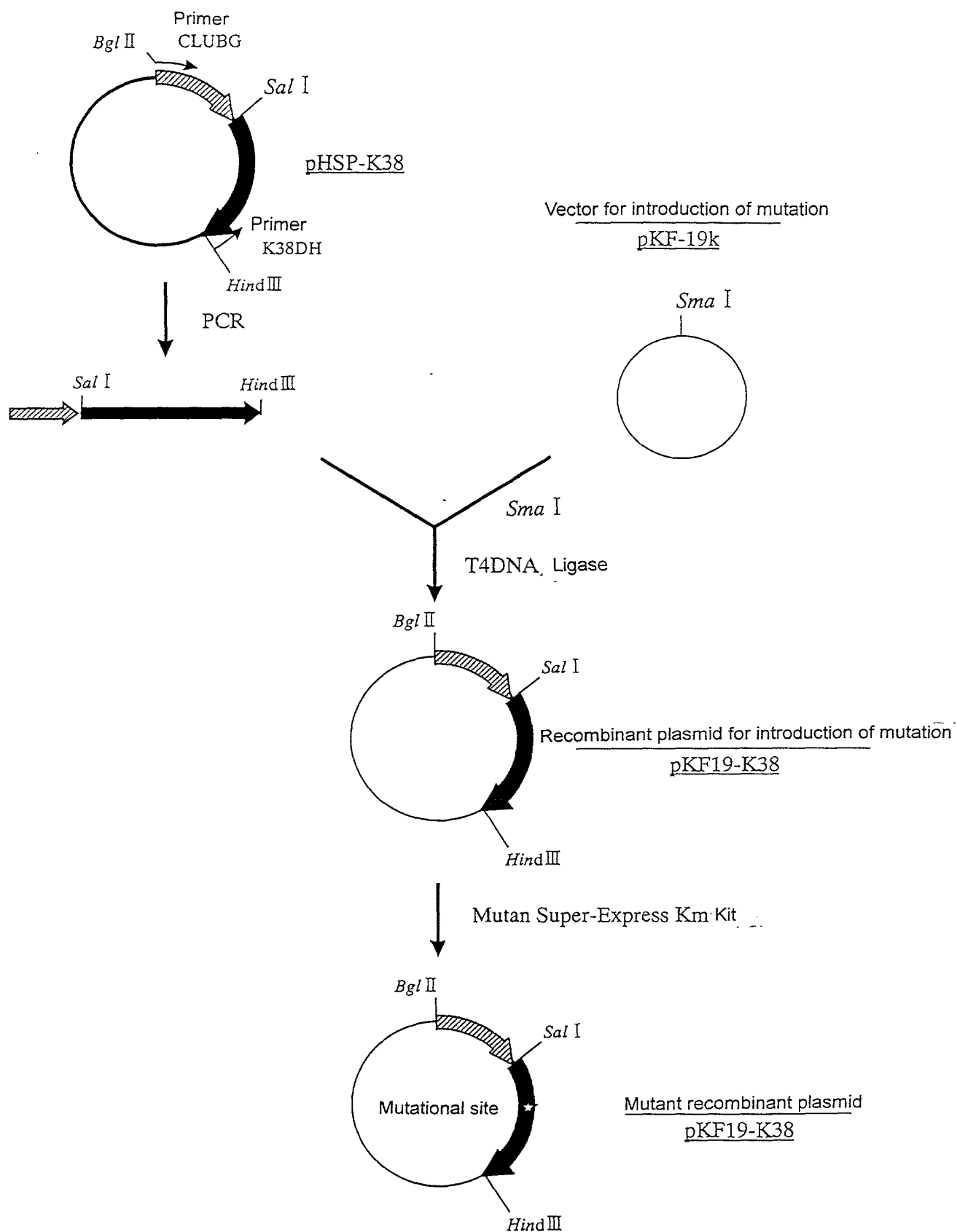
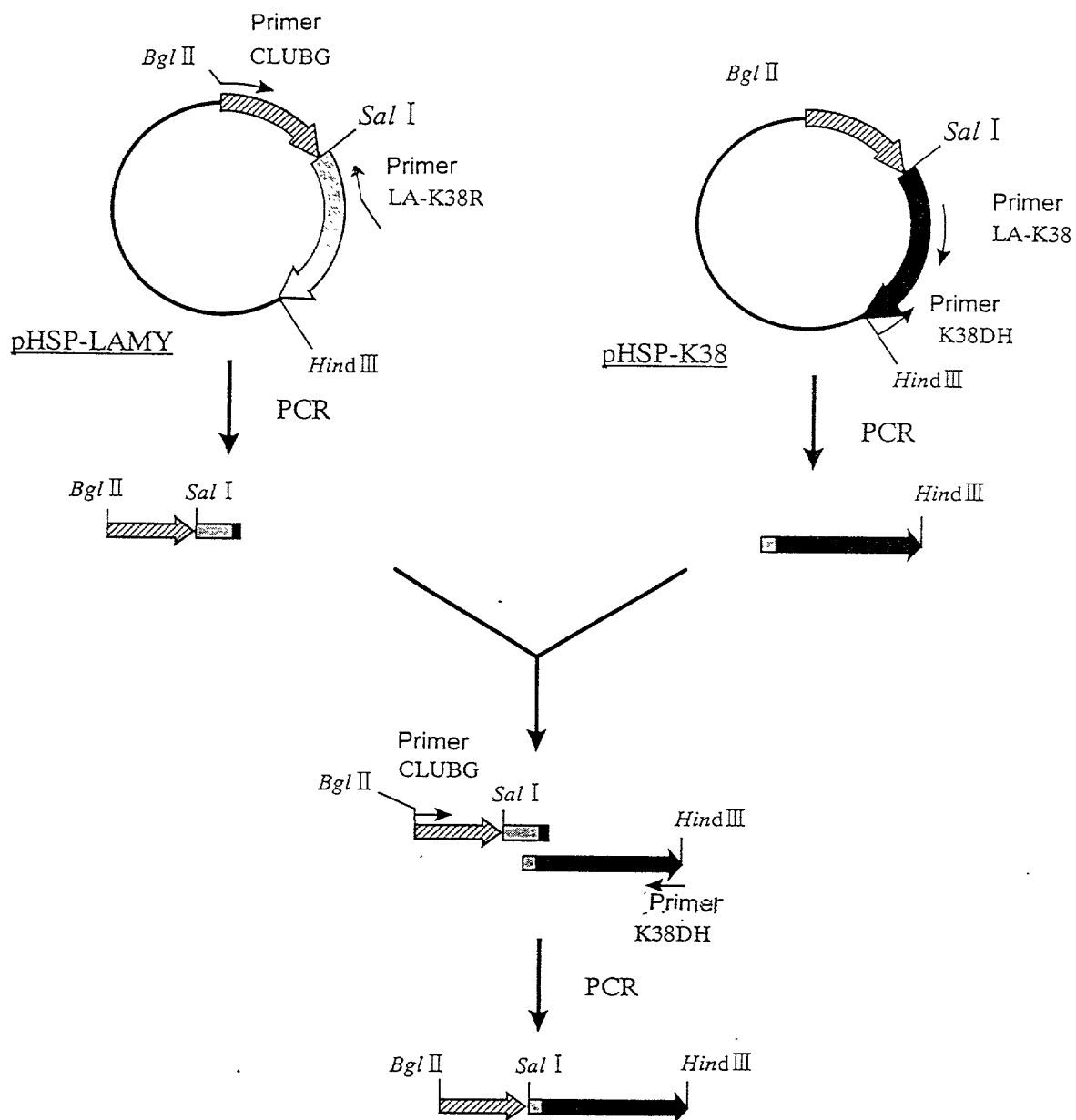


Fig. 3



# BIRCH, STEWART, KOLASCH & BIRCH, LLP

PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:

## COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT AND DESIGN APPLICATIONS

ATTORNEY DOCKET NO.

2173-0120P

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor ( if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title:

MUTANT  $\alpha$ -AMYLASES

Fill in Appropriate  
Information -  
For Use Without  
Specification  
Attached:

the specification of which is attached hereto. If not attached hereto,

the specification was filed on \_\_\_\_\_ as  
United States Application Number \_\_\_\_\_; and /or

the specification was filed on \_\_\_\_\_ as PCT  
International Application Number \_\_\_\_\_; and was  
amended under PCT Article 19 on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority  
Information:  
(if appropriate)

Prior Foreign Application(s)

163569/1999

(Number)

Japan

(Country)

June/10/1999

(Month/Day/Year Filed)

Priority Claimed

☒ Yes

☐ No

☐ Yes

☐ No

☐ Yes

☐ No

☐ Yes

☐ No

☐ Yes

☐ No

☐ Yes

☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

Insert Provisional  
Application(s):  
(if any)

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months (6 Months for Designs) Prior To The Filing Date of This Application:

Insert Requested  
Information:  
(if appropriate)

Country

Application No.

Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Insert Prior U.S.  
Application(s):  
(if any)

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)



I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

Terrell C. Birch (Reg. No. 19,382)  
Joseph A. Kolasch (Reg. No. 22,463)  
Bernard L. Sweeney (Reg. No. 24,448)  
Charles Gorenstein (Reg. No. 29,271)  
Leonard R. Svensson (Reg. No. 30,330)  
Andrew D. Meikle (Reg. No. 32,868)  
Joe McKinney Muncy (Reg. No. 32,334)  
C. Joseph Faraci (Reg. No. 32,350)

Raymond C. Stewart (Reg. No. 21,066)  
James M. Slattery (Reg. No. 28,380)  
Michael K. Mutter (Reg. No. 29,680)  
Gerald M. Murphy, Jr. (Reg. No. 28,977)  
Terry L. Clark (Reg. No. 32,644)  
Marc S. Weiner (Reg. No. 32,181)  
Andrew F. Reish (Reg. No. 33,443)  
Donald J. Daley (Reg. No. 34,313)

Send Correspondence to:

**BIRCH, STEWART, KOLASCH & BIRCH, LLP**

**P.O. Box 747 • Falls Church, Virginia 22040-0747**

**Telephone: (703) 205-8000 • Facsimile: (703) 205-8050**

PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole  
Inventor:  
Insert Name of Inventor  
Insert Date This  
Document is Signed

Insert Residence  
Insert Citizenship

Insert Post Office  
Address

Full Name of Second  
Inventor, if any:

see above

Full Name of Third  
Inventor, if any

see above

Full Name of Fourth  
Inventor, if any

see above

Full Name of Fifth  
Inventor, if any

see above

GIVEN NAME Keiji	FAMILY NAME ENDO	INVENTOR'S SIGNATURE Keiji Endo	DATE* May 29, 2000
Residence (City, State & Country) Haga-gun, Tochigi 321-3497 Japan		CITIZENSHIP Japanese	
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Kao Corporation, Research Laboratories, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497 Japan			
GIVEN NAME Kazuaki	FAMILY NAME IGARASHI	INVENTOR'S SIGNATURE Kazuaki Igarashi	DATE* May 29, 2000
Residence (City, State & Country) Haga-gun, Tochigi 321-3497 Japan		CITIZENSHIP Japanese	
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Kao Corporation, Research Laboratories, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497 Japan			
GIVEN NAME Yasuhiro	FAMILY NAME HAYASHI	INVENTOR'S SIGNATURE Yasuhiro Hayashi	DATE* May 29, 2000
Residence (City, State & Country) Haga-gun, Tochigi 321-3497 Japan		CITIZENSHIP Japanese	
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Kao Corporation, Research Laboratories, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497 Japan			
GIVEN NAME Hiroshi	FAMILY NAME HAGIHARA	INVENTOR'S SIGNATURE Hiroshi Hagihara	DATE* May 29, 2000
Residence (City, State & Country) Haga-gun, Tochigi 321-3497 Japan		CITIZENSHIP Japanese	
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Kao Corporation, Research Laboratories, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497 Japan			
GIVEN NAME Katsuya	FAMILY NAME OZAKI	INVENTOR'S SIGNATURE Katsuya Ozaki	DATE* May 29, 2000
Residence (City, State & Country) Haga-gun, Tochigi 321-3497 Japan		CITIZENSHIP Japanese	
POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Kao Corporation, Research Laboratories, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497 Japan			

\* DATE OF SIGNATURE